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DELIA M. PINTO

Ozonolysis of Constitutively-emitted and Herbivory-induced Volatile Organic Compounds (VOCs) from Plants

Consequences in Multitrophic Interactions

Doctoral dissertation

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ABSTRACT

Herbivore feeding on plant tissue induces the emission of phytogenic volatile organic compounds (VOCs) that differ either quantitatively or qualitatively from those emitted constitutively and after mechanical damage. Natural enemies of herbivores, e.g. carnivorous arthropods that predate or parasitise, have learned to exploit herbivore-induced VOCs to find suitable preys or hosts. Although individual compounds are enough to attract natural enemies, in nature they might utilise ratios of various compounds.

Herbivore-induced VOC blends include an array of terpenes as well as aldehydes, esters and alcohols, commonly referred to as green leaf volatiles (GLVs), which have been shown to attract natural enemies. Terpenes and GLVs can be readily degraded by atmospheric ozone (O_3) which reacts with C-C double bonds. Hence, it has been suggested that predicted increases of tropospheric O_3, as a result of anthropogenic activity, may disrupt the searching behaviour of natural enemies.

The main objectives of the present study were to assess the effects of O_3 on the relative proportions of constitutively-emitted and herbivore-induced VOCs, and to assess whether these changes affect the orientation of natural enemies. For this purpose, a system was developed that allowed the collection and analysis of headspace VOCs at different O_3 concentrations in laboratory chambers with controlled atmospheres. In addition, the system allowed the assessment of natural enemy behavioural responses, which were assessed in two tritrophic systems (*Brassica oleracea-Plutella xylostella-Cotesia vestalis (=C. plutellae)* and *Phaseolus lunatus-Tetranycus urticae-Phytoseiulus persimilis*). To support the laboratory experiments, a two-year field trial was conducted assessing the orientation of the parasitoid *C. vestalis* in double background O_3 concentration.

The results showed that near ambient and elevated O_3 concentrations lead to drastic changes in the VOC blends, altering the concentrations and the relative proportions of VOCs. Monoterpenes were degraded and when reacting with very high O_3 concentrations (200 and 400 ppbv) had the potential to form secondary aerosols. Very reactive inducible terpenes such as sesqui-, and homoterpenes may become undetectable at moderately elevated O_3 concentrations (60 and 120 ppbv) reflecting what we could realistically expect in the future. Despite the degradation of terpenes and GLVs by O_3, natural enemies were able to orientate towards herbivore-damaged plants. In field conditions, the host location and parasitism rates of *C. vestalis* were also unaffected. Less reactive induced compounds (other than terpenes and GLVs) were identified. An additional laboratory experiment showed that *C. vestalis* orientates toward a non-degraded herbivore-induced VOC blend over an oxidised one.

In conclusion, results with the model systems suggest that O_3-enriched conditions might not disrupt the orientation of natural enemies towards their herbivore prey or host. The herbivore-induced VOC blend includes compounds that are not affected by O_3 and yet may act as infochemicals for natural enemies. As O_3 and other oxidants are currently present in the atmosphere, my hypothesis is that these less reactive compounds act as long-distance infochemicals, whereas terpenes and GLVs are used in short-distance communication. Oxidation products resulting from the ozonolysis of reactive compounds seem not to play a role in the orientation of natural enemies, as shown for *C. vestalis.*

Universal Decimal Classification: 546.214, 547.596, 581.116, 632.937.1
CAB Thesaurus: volatile compounds; organic compounds; chemical degradation; ozone; plants; leaves; herbivores; terpenoids; natural enemies; orientation; trophic levels; *Cotesia plutellae; Phytoseiulus persimilis*

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Plants used in the experiments were not just a few! I want to express my gratitude to the staff of the Research Garden for growing and watering the cabbage plants I used for the experiments and other tests.

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Kuopio, September 2008

Delia M. Pinto

ABBREVIATIONS

BC	benzyl cyanide
DMAPP	dimethylallyl diphosphate
DMNT	(*E*)-4,8-dimethyl-1,3,7-nonatriene
DMPS	differential mobility particle sizer
GC-MS	gas chromatography – mass spectrometry
GLV	green leaf volatile
IPP	isopentenyl diphosphate
KI	potassium iodide
LOX	lipoxygenase
MeJA	methyl jasmonate
MeSA	methyl salicylate
MEP	methyl-erythritol phosphate
MVA	mevalonic acid
NO_X	nitrogen oxides
NO_2	nitrogen dioxide
$NO_3\cdot$	nitrate radical
ng l^{-1}	nanograms per liter
ng g^{-1} DW h^{-1}	nanograms per gram of dry weight per hour
nl l^{-1}	nanoliters per liter
$OH\cdot$	hydroxyl radical
O_3	ozone
ppbv	parts per billion per volume
ROS	reactive oxygen species
SOAs	secondary organic aerosols
TMTT	(*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene
VOC	volatile organic compound

LIST OF ORIGINAL PAPERS

This thesis is based on the following publications, which are referred to in the text by their chapter numbers:

Chapter 2 Pinto DM, Tiiva P, Miettinen P, Joutsensaari J, Kokkola H, Nerg A-M, Laaksonen A, Holopainen JK. 2007. The effects of increasing atmospheric ozone on biogenic monoterpene profiles and the formation of secondary aerosols. Atmospheric Environment 41: 4877-4887

Chapter 3 Pinto DM, Blande JD, Nykänen R, Dong, WX, Nerg A-M, Holopainen JK. 2007. Ozone degrades common herbivore-induced plant volatiles: does this affect herbivore prey location by predators and parasitoids? Journal of Chemical Ecology 33: 683-694

Chapter 4 Pinto DM, Nerg A-M, Holopainen JK. 2007. The role of ozone reactive compounds, terpenes and green leaf volatiles (GLVs), in the orientation of *Cotesia plutellae*. Journal of Chemical Ecology 33: 2218-2228

Chapter 5 Pinto DM, Himanen S, Nissinen A, Nerg A-M, Holopainen JK. 2008. Host location behavior of *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) in ambient and moderately elevated ozone in field conditions. Environmental Pollution doi:10.1016/j.envpol.2007.12.009

CONTENTS

CHAPTER 1

General Introduction

CHAPTER 1: General Introduction

1.1 Importance of the Study – Climate change and the ecosystems

Anthropogenic activities such as burning of fossil fuels, deforestation, agricultural activities (e.g. fertilization), and livestock activities are resulting in increased concentrations of greenhouse gases in the atmosphere. Greenhouse gases such as carbon dioxide (CO_2) and tropospheric ozone (O_3), as well as increased surface temperatures as a result of the greenhouse effect (IPCC 2007), have been predicted to have a negative impact on future agroecosystems (Fuhrer 2003 for a review). In the last two decades, many studies have focused on the effects of these greenhouse gases on plants and the second trophic level (Valkama et al 2007 and references therein). However, few have investigated the direct and/or plant mediated effects of these gases on higher trophic levels e.g. natural enemies of herbivores (Gate et al 1995; Percy et al 2002). Assessing the effects of these gases on higher trophic levels is important since natural enemies play an important role in population dynamics of herbivores (Percy et al 2002). It has been speculated that O_3 can affect the behaviour of foraging natural enemies since it can chemically degrade and transform volatile organic compounds (VOCs) acknowledged to act as olfactory cues (Gate et al 1995). To my knowledge this is the first time that the effects of O_3 on VOCs and the consequences for orientation of foraging natural enemies have been addressed.

1.2 Phytogenic Volatile Organic Compounds (VOCs)

Plants emit substantial amounts of volatile organic compounds (VOCs) to the atmosphere. Volatilities of these compounds are determined by their low molecular weights and high vapour pressures (Raguso 2004; Dudareva et al 2006). It has been estimated that global phytogenic VOC emissions are in the order of $1,2 \times 10^{15}$ g C per year, which exceeds those from anthropogenic sources (Guenther et al 1995). Phytogenic VOCs include isoprenoids (isoprene and terpenoids), alkanes, alkenes, alcohols, aldehydes, eters, esters and acids. Isoprenoids are among the most abundant of these, followed by alcohols and carbonyls (Kesselmeier & Staudt 1999).

1.2.1 Biosynthesis, Storage and Emission of VOCs

Although there are few biochemical pathways involved in the synthesis of VOCs, a total of 1700 volatile compounds have been described. Such diversity is the result of enzymatic modifications (Dudareva et al 2006 for a review). VOCs with similar hydrocarbon skeletons may vary in their degree of unsaturation, functional group or oxidative state (Raguso 2004).

Plant VOCs can be divided into three major groups: isoprenoids (isoprene and terpenoids), fatty acid derivates and phenylpropanoids/benzenoids. **Isoprenoids** form a large group of compounds that include hormones such as abscisic acid and gibberellic acid, membrane components such as sterols, and photosynthetic pigments such as carotenoids and chlorophylls, which are vital for the functioning of the plant (Eisenreich et al 2004; Owen & Peñuelas 2005). They also comprise the largest group of plant-emitted VOCs. The biosynthetic pathway of this group has been widely studied and recently reviewed by Dudareva et al (2006). In brief, they originate from five-carbon precursors: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Pichersky et al 2006). The biosynthesis of isoprenoids occurs in two different cellular compartments: the cytosol and the plastid. In the cytosol, IPP is synthesised from three molecules of acetyl-CoA via the mevalonic acid (MVA) pathway (Dudareva et al 2006; Pichersky et al 2006). Thereafter, the enzyme farnesyl pyrophosphate synthase catalyses the condensation of two molecules of IPP and one molecule of DMAPP to form farnesyl pyrophosphate (FPP), the precursor of sesquiterpenes (Dudareva et al 2006). In the plastid, IPP is derived from pyruvate and glyceraldehyde-3-phosphate via the methyl-erythritol-phosphate (MEP) pathway (see Eisenreich et al 2004 for a review of the MEP pathway). The allyl isomer is used in the synthesis of isoprene. The enzyme geranyl pyrophosphate synthase catalyses the condensation of one molecule of IPP and one molecule of DMAPP to form geranyl pyrophosphate (GPP), the precursor of monoterpenes. In addition, the geranylgeranyl pyrophosphate synthase catalyses the condensation of one molecule of DMAPP with three molecules of IPP to form geranylgeranyl pyrophosphate (GGPP) (Dudareva et al 2006; Pichersky et al 2006), the precursor of diterpenes. Thereafter, terpene synthases are responsible for producing the diverse range of terpenes (Dudareva et al 2006). This group of enzymes catalyses the formation of a carbocation (unstable

intermediate) from pyrophosphates (DMAPP, GPP, FPP and GGPP) by removing the pyrophosphate group and terpenes are formed by subsequent transformations (Pichersky et al 2006). Additionally, some terpenes can be formed through additional modification reactions such as hydroxylation, dehydrogenation, acylation, etc. Additional modification reactions also lead to the formation of irregular homoterpenes (Dudareva et al 2006).

The second group of phytogenic VOCs are the **volatile fatty acid derivates.** As the name of the group suggests, these compounds originate in cell membrane-derived fatty acids: linolenic acid or linoleic acid, which are oxygenated via the lipoxygenase (LOX) pathway. Oxidation by different LOXs results in the formation of two groups: the 9-hydroxyperoxy and 13-hydroxyperoxy derivates of polyenoic fatty acids. Thereafter, they can be cleaved in reactions catalysed by a hydroperoxide lyase resulting in short chain C6 or C9 volatile compounds (Dudareva et al 2006). Alternatively, they can be metabolised by other enzymes, which is the case with methyl jasmonate (MeJA). This compound is formed via the octadecanoid pathway and is derived from 13-hydroperoxy-linolenic acid, which forms allene oxide in dehydrase-catalysed reactions. Further cyclizations and oxidations of allene oxide lead to the formation of jasmonate acid (Karban & Baldwin 1997; see Schaller 2001 for a review of the biosynthesis of octadecanoid-derived molecules). The final stage in the formation of the volatile methyl ester MeJA is catalysed by a methyl transferase (Duradeva et al 2006).

The third group of plant VOCs are the **phenylpropanoids/benzenoids**. These compounds play important roles in plant defence and reproduction (Dixon et al 2002; Dudareva et al 2006). The biosynthetic pathways responsible for their formation have been little studied so far. L-phenylalanine, synthesised via the shikimic acid pathway is converted into (*E*)-cinnamic acid in reactions catalysed by L-phenylalanine ammonia-lyase (PAL), an enzyme responsible for deamination (Dixon et al 2002; Dudareva et al 2006). In further steps of phenylpropanoid synthesis, the (*E*)-cinnamic is converted into a diverse range of hydroxycinnamic acids, aldehydes and alcohols, which include primary and secondary non-volatile compounds (Dixon et al 2002; Dudareva et al 2006), which in turn can form volatile compounds (Dudareva et al 2006; Pichersky et al 2006). Benzenoid compounds such as benzoic acid and

benzaldehyde, are formed by the shortening of the (*E*)-cinnamic side chain (Dudareva et al 2006; Pichersky et al 2006). Salicylic acid (SA) and its volatile methyl ester methyl salicylate (MeSA) are formed via this route (Lee et al 1995; Dixon et al 2002) and are important compounds in plant defence. Besides L-phenylalanine, other amino acids such as alanine, valine, leucine, isoleucine and methionine are also precursors for aldehydes, alcohols, esters, acids, and nitrogen and sulfur-containing volatiles (Duradeva et al 2006). For example, glucosinolates, an important group of secondary metabolites in plants of the order Brassicales, are biosynthesised from tryptophan and seven additional amino acid precursors (Grubb & Abel 2006; Yan & Chen 2007, for a review on glucosinolate synthesis). The role of the volatile glucosinolate breakdown products will be discussed later (Chapter 6). Finally, some volatile compounds are synthesised as a product of carotenoid cleavage by carotenoid cleavage dioxygenases (Pichersky et al 2006). After synthesis, plant VOCs can be stored either in specialised structures or within leaves. In the latter case, compounds are stored in the lipid or the aqueous phase of the leaf depending on whether they are lipo- or hydrophilic (Niinemets et al 2004).

VOC emission by all plant species is regulated by genetic factors (Peñuelas & Llusià 2001). For instance, not all plant species emit isoprene, and although terpenes and short chain aldehydes and alcohols are widely distributed in the plant kingdom, the chemical nature of the compounds varies. Besides genetic factors, temperature and light affect their synthesis, by affecting the availability of precursors and energy capacity (Niinemets et al 2004), which in turn affects the size of the VOC pools (Peñuelas & Llusià 2001 for a review).

Emission to the atmosphere occurs by diffusion from leaves. This means that temperature also affects emission rates by increasing the volatility and diffusion rates (Niinemets et al 2004). Additionally, in the short term (minutes to hours) light impacts on the emission rates of non-stored VOCs that need photosynthetic products for their biosynthesis (Peñuelas & Llusià 2001; Niinemets et al 2004). Whereas compounds with the highest volatility are the most responsive to temperature, compounds with the lowest volatility are the least responsive to irradiance (Peñuelas & Llusià 2001).

1.2.2 Functions of Phytogenic VOCs

Many eco-physiological functions have been attributed to phytogenic VOCs (Fig. 1). They offer defence against different stressors and drive communication between the plant and its biotic environment. They protect the plant against abiotic stresses such as oxidation, by scavenging reactive oxygen species (ROS) and quenching ozone (O_3) from the atmosphere (Loreto et al 2001; Loreto et al 2004). They also protect the plant from heat by scavenging ROS produced by high temperatures (Peñuelas & Llusià 2003). In addition, they protect plants from numerous fungi and bacteria, and repel insects (Dudareva et al 2006 for a review).

VOCs also drive plant-animal interactions, which are of paramount importance since they bind food webs within complex ecosystems and in some cases determine agricultural productivity (Raguso 2004). VOCs mediate the attraction of pollinators to flowers (Raguso 2004) and of animal seed dispersers to odorous fruits (Dudareva et al 2006). They also mediate host location by herbivorous insects (Bruce et al 2005) and the recruitment of natural enemies in many tritrophic systems e.g. predatory and parasitic arthropods above ground (Dicke 1999, for a review), as well as entomopathogenic nematodes below-ground (van Tol et al 2001; Rasmann et al 2005).

VOCs seem to be also involved in plant-plant interactions (Dicke et al 2003 for a review ; Karban et al 2003). Although there is still some scepticism, there is evidence that VOCs drive communication between plants. VOCs released from damaged plants elicit defensive responses in undamaged neighbouring plants that include gene activation (for example Arimura et al 2000; Arimura et al 2001). They prime undamaged neighbouring plants to respond to further attacks (Choh & Takabayashi, 2006a). Moreover, intact plants exposed to herbivore-induced volatiles become attractive to natural enemies (Choh & Takabayashi, 2006b).

In addition to their eco-physiological functions, VOCs emitted by vegetation, particularly forests, also play an important role in atmospheric chemistry. In the presence of NO, emitted from combustion sources, they are involved in a series of photochemical reactions contributing to the formation of tropospheric ozone (O_3)

(Atkinson & Arey 2003). The implications of this atmospheric oxidant on terrestrial ecosystems will be discussed later (1.4). Moreover, VOCs contribute to the formation of secondary organic aerosols (SOAs) (Seinfeld & Pandis 2006), and therefore, of cloud condensation nuclei, which can have a dramatic effect on climate (Peñuelas & Llusià 2003).

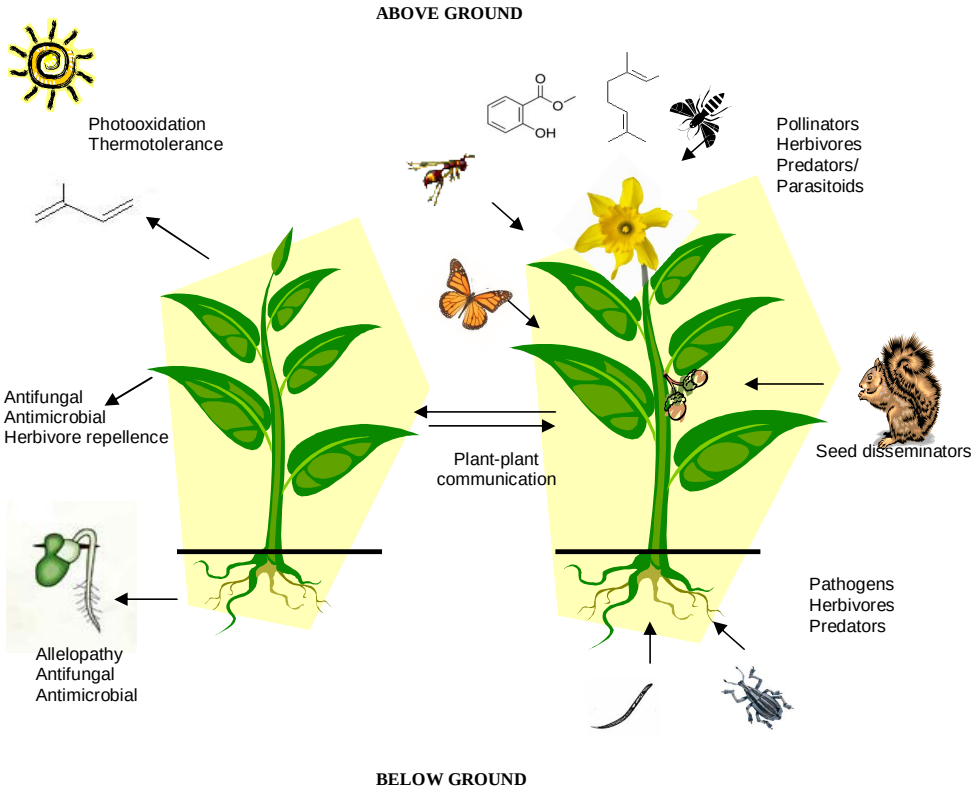

Figure 1. Eco-physiological functions of plant-emitted VOCs modified from Dudareva et al (2006)

1.2.3 Reactivity and gas-phase reaction of VOCs

The isoprenoid group have been the target of many studies in the field of atmospheric chemistry, although other compounds emitted by plants including alcohols have also been studied (Aschmann et al 1997). The lifetimes of many isoprenoids and some oxygenated compounds when exposed to atmospheric oxidants such as OH·, O_3 and NO_3· have been reviewed by Atkinson & Arey (2003). Lifetimes can vary from a few

minutes to hours and even days and years depending on which oxidant they react with. For example, the lifetimes of the monoterpene β-pinene are in the order of 1.8 hours, 1.1 day and 27 minutes when reacting with OH· (assumed concentration 2,0 x 10^6 molecule cm^{-3}, 12-h daytime average), O_3 (assumed concentration 7,0 x 10^{11} molecule cm^{-3}, 24-h average) and NO_3· (assumed concentration 2,5 x 10^8 molecule cm^{-3}, 12-h night time average), respectively. In the specific case of monoterpene reactions with O_3, there is great variation among compounds and the lifetimes can vary between minutes and days (Calogirou et al 1999). For example, the lifetime of α-terpinene is just one minute, whereas the lifetime of 1,8-cineole is greater than 110 days (assumed concentration 7,0 x 10^{11} molecule cm^{-3}, 24-h average) (Atkinson & Arey 2003). The variation in the reactivity of terpenoids depends on their chemical structure. Saturated compounds such as 1,8-cineole and camphor are not affected by O_3; compounds with one C-C double bond such as camphene, α- and β-pinene, and sabinene are slightly decomposed; and polyunsaturated compounds such as d-limonene, β-ocimene, and α-terpinene are significantly affected by O_3 (Calogirou et al 1996).

The gas phase oxidation of terpenes has been reviewed by Calogirou et al (1999). Their reaction with atmospheric oxidants e.g. OH·, O_3 and NO_3 results in a series of compounds in both gas and particulate phases (Calogirou et al 1999; Yu et al 1999; Joutsensaari et al 2005). When oxidised by O_3, the main products observed are compounds from the carbonyl group (mainly ketones, hydroxyketones and aldehydes) as well as carboxylic acids (Calogirou et al 1999; Yu et al 1999). In brief, the ozonolysis of terpenes is as follows: O_3 adds to the double bond(s) of terpenes leading to the formation of a primary unstable ozonide. The ozonide decomposes to form epoxides as well as an energy-rich biradical (Criegee intermediate) and a carbonyl (Calogirou et al 1999). Further decomposition of the excited Criegee intermediate also results in the formation of OH· radicals and organic acids (Pfeiffer et al 1998; Aschmann et al 2002). A series of reactions leads to stabilised products. The stabilised Criegee intermediates may react with water molecules resulting in the formation of organic acids (Calogirou et al 1999; Seinfeld & Pandis 2006) Aldehydes and hydrogen peroxide can also be formed in side-reactions (Calogirou et al 1999).

Some products of VOC oxidation are compounds with low volatility that form new particles or condense onto pre-existing ones in order to establish equilibrium between the gas and aerosol phases (gas/phase partitioning) (Seinfeld & Pandis 2006). This is the case with carboxylic acids, which result from reactions between O_3 and several terpenes (Hoffmann et al 1998; Yu et al 1999). One factor that influences the potential of terpenes to form SOAs is their chemical structure (Hoffmann et al 1997; Lee et al 2006). Hoffmann et al (1997) found that compounds with several C-C double bonds, such as limonene, can produce more particles than cyclic terpenes such as α-pinene and Δ-3-carene, which in turn can produce more particles than acyclic compounds. Similarly, in a study comparing the potential of terpenes to produce SOAs, Lee et al (2006) found that compounds with one or more double bonds internal to the ring structure had the greatest potential to form SOAs. Although the potentials of isoprene and monoterpenes for forming SOAs have been the subjects of many studies (Hoffmann et al 1998; Yu et al 1999; Claeys et al 2004), the evidence that sesquiterpenes could be a major source of particles in the atmosphere is accumulating (Bonn and Moortgat, 2003; van Reken et al 2006). Sesquiterpenes might contribute to the nucleation process through the rapid formation of condensable products (Calogirou et al, 1999; Lee et al 2006).

1.3 Indirect Defence of Plants – Tritrophic Signalling

The term indirect defence of plants refers to the emission of inducible VOCs by plants in response to herbivory, which are exploited by natural enemies, e.g. predatory and parasitic arthropods (Dicke 1999), and entomopathogenic nematodes (van Tol et al 2001; Rasmann et al 2005) that exert "top-down" control (Herbivore population densities are regulated by higher trophic levels). This contrasts with "bottom-up" control strategies in which plant resources are used (Power 1992). From an agricultural point of view, knowledge about indirect defence of plants can be exploited to improve biological control of crops (Turlings & Wäckers 2004). From an ecological point of view, inducible VOCs drive the signalling between three different trophic levels: plants, herbivores and carnivores, and to an extent play an important role in population dynamics (Dicke 1999). Although it is not clear how indirect defence of plants has evolved, it offers a great advantage for both plants and carnivores, since herbivores are under constant selection to avoid encountering their

natural enemies (Turlings & Wäckers 2004). As well as herbivore feeding, herbivore oviposition has also been shown to drive tritrophic signalling (Hilker & Meiners 2006). The exploitation of herbivore-induced VOCs has been documented in a large number of tritrophic systems (Dicke 1999, for a review).

1.3.1 Compounds involved in Indirect Defence

Herbivore-induced volatiles include an array of alcohols, aldehydes and esters, synthesised via the LOX pathway, and many terpenoids (Section 1.2.1). Compounds derived from the LOX pathway are commonly referred to as green leaf volatiles (GLVs) and can account for more than 50% of emissions from damaged parts of some plant species (Holopainen 2004). These emissions are the result of mechanical cell damage, and therefore, take place soon after the onset of herbivore feeding (Holopainen 2004; Scascighini et al 2005). They are considered to be passively released. However, there is some specificity in the ratios of GLVs from the same plant species when attacked by two different herbivores (Turlings & Wäckers 2004). They evoke electrophysiological (Smid et al 2002) and behavioural responses (Reddy et al 2002) in natural enemies. This suggests that GLVs are involved in the indirect defence of plants. In addition, they are able to trigger defence responses in neighbouring plants such as accumulation of phytoalexins and emission of VOCs (Engelberth et al 2004; Ruther & Kleier 2005; Yan & Wang 2006). They also induce the secretion of extrafloral nectar (Ruther & Kleier 2005; Kost & Heil 2006), which is another type of induced defence (Turlings & Wäckers 2004). The activation of LOX and PAL genes has also been observed after exposure of plants to (*Z*)-3-hexenol (Paré et al 2005, for a review).

The second major group of compounds induced by herbivory are terpenoids, which tend to dominate the herbivore-induced VOC blend of some species, e.g. maize (Turlings & Ton 2006). Besides constitutively-emitted terpenes, whose emissions can be greatly enhanced by herbivory, the herbivore-induced VOC blend also includes terpenes (mono-, sesqui-, and homoterpenes) that are synthesised *de novo* (Paré & Tumlinson 1999). These inducible compounds have been acknowledged as important synomones, e.g. infochemical benefiting both the emitter and the receiver (Holopainen 2004; Turlings & Ton 2006). The emission of inducible compounds

varies among plant species. However, two inducible acyclic homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) are induced in many plant species (Karban & Baldwin 1997).

Besides GLVs and terpenes, herbivory induces the emission of other compounds. For instance, herbivory induces the emission of methyl salicylate (MeSA). This methyl ester is a product of metabolism of salicylic acid, which is synthesised from cinnamic acid (Lee et al 1995). Although salicylic acid has mainly been shown to be involved in the systemic acquired resistance of plants to pathogens (Lee et al 1995), its volatile ester is induced after mite feeding, and it is now clear that it has a relevant role in the recruitment of predatory mites (De Boer & Dicke 2004). Indole, synthesised via the shikimic acid pathway, is another compound that is induced by herbivore feeding in some plant species such as cotton and maize (Paré & Tumlinson, 1999; Turlings & Wäckers 2004). Others can be emitted by specific taxonomic plant groups. For example, herbivory induces the emission of volatile glucosinolate breakdown products in cruciferous species (Mattiacci et al 1994; Schoonhoven et al 2006)

1.3.2 Biochemical and Genetic Evidence

Indirect defences imply the activation of well orchestrated biochemical pathways. Van Poecke & Dicke (2002) found that the attraction of a parasitoid to *Arabidopsis* plants involves the activation of both the octadecanoid and the salicylic acid pathways. In a first step, indirect defence is triggered by herbivore-derived elicitors. The herbivore-induced VOC blend cannot be mimicked by mechanical damage alone, but it can be by the addition of regurgitant or elicitors isolated from the oral secretion of insects (Mattiacci et al 1995; Alborn et al 1997). These elicitors can be classified in two major groups: the **lytic enzyme group** which includes β-glucosidases, glucose oxidases and alkaline phosphatases, and the **fatty acid-amino conjugates** (Paré et al 2005). The most well studied examples are the lytic enzyme β-glucosidase, which is present in the regurgitant of *Pieris brassicae* and elicits the emission of VOCs from cruciferous plants (Mattiacci et al 1995); and the fatty acid-amino acid conjugate N-(17-hydroxylinolenoyl)-L-glutamine (volicitin), which is present in the oral secretion

of *Spodoptera exigua* and triggers the emission of VOCs from maize seedlings (Alborn et al 1997).

Plant defence responses, including the induction of VOC emissions, are the result of the activation of specific downstream signal transduction pathways by chemical elicitors (Paré et al 2005). For instance, volicitin activates the gene *igl* that encodes the synthesis of the enzyme indole-3-glycerol phosphate lyase which in turn synthesises free indole from its precursor indole-3-glycerol phosphate (Turlings & Wäckers 2004, for a review). Indole is the main shikimic-acid-derived compound induced after herbivore feeding in maize, even though it seems not to play a role in the orientation of two parasitoids (D'Alessandro et al 2006). Volicitin also activates the gene *stc1* that encodes a specific maize sesquiterpene cyclase. Feeding by *Spodoptera littoralis* on maize and spider mites on lima bean leads to the activation of the enzyme (*E*)-nerolidol synthase, which is involved in the synthesis of the herbivore-induced homoterpene DMNT (Turlings & Wäckers 2004 for a review).

1.3.3 Variability and Specificity

Herbivore-induced VOC profiles show great variability. They vary according to plant species (van den Boom 2004), and attacking herbivore (De Moraes et al 1998). Different profiles are also emitted in response to feeding by herbivores at different developmental stages (Takabayashi et al 1995). Variation between VOC blends has also been observed between genotypes, within plant parts of the same individual and in time (Turlings & Wäckers 2004, for a review). Abiotic factors such as air and soil humidity, temperature, light intensity, photoperiod and fertilization rate can also affect either the quality or quantity of the blend (Gouinguené & Turlings 2002). Furthermore, the growth stage of the plant is another factor that affects the herbivore-induced VOC blend (Turlings & Wäckers 2004). Hence, variation provides natural enemies with information not only about the herbivore but also about the attacked plant and its physiological state. In addition to herbivory, VOCs are induced in response to wounding (Mattiaci et al 1994), environmental pollutants (Vuorinen et al 2004), and exogenous plant-derived (Dicke et al 1999) as well as pathogen-derived (Turlings & Wäckers 2004 for a review) elicitors. Although similar, the VOC profiles

induced by different elicitors vary to a certain extent which allows natural enemies to orientate towards those induced by herbivores (e.g. Vuorinen et al 2004).

Specificity between the phytogenic herbivore-induced VOC blend and natural enemies has been observed in many studies (Takabayashi et al 1995; De Moraes et al 1998), although lack of specificity has also been reported (Gouinguené et al 2003). The specificity of the blend is important for the orientation of natural enemies to their most suitable host or prey. It is especially important for specialist or oligophagous natural enemies (Turlings & Wäckers 2004, for a review). The VOC blend induced by herbivory can differ either quantitatively (increased amounts of the same compounds) or qualitatively (induction of novel compounds) from the one induced by mechanical damage (Dicke 1999). These differences in quality or quantity offer natural enemies reliable information about the presence of a suitable host or prey. For instance, the attraction of parasitic wasps to herbivore-damaged maize seems to rely more on qualitative than quantitative differences in the VOC blend (Fritzsche Hoballah et al 2002). Since different plant species can share the same compounds in their VOC profiles, it has been proposed that the olfactory orientation of insects is based on compound ratios (Bruce et al 2005). In addition, only a part of the VOC blend might offer information to natural enemies (De Boer & Dicke 2005), since a plant can emit over a hundred compounds in the same blend.

1.4 Ozone

O_3 plays an important role in atmospheric chemistry. In the upper stratosphere, O_3 protects the Earth from the sun's harmful ultraviolet radiation. Therefore, stratospheric O_3 could be referred to as "good O_3". In the lower troposphere, low concentrations of O_3 are also present as a result of natural sources (Vingarzan 2004). However, increased concentrations can be harmful to ecosystems and hence, it could be referred to as "bad O_3". Human activity is leading to the depletion of the "good O_3" and to an increase in the "bad O_3". The latter is the result of increases in industrial emissions of O_3 precursors, e.g. methane, nitrogen oxides (NO_x), carbon monoxide and VOCs (IPCC, 2007), among which the emissions of NO_x during fossil-fuel burning are the major anthropogenic source (Vingarzan 2004). Tropospheric O_3 is becoming an important greenhouse gas (IPCC 2001). It has been estimated that O_3 increases

between 0.5 and 2% per year (Vingarzan 2004), and it has been projected that by 2100 its mean monthly 24-h concentration will be above 40 ppbv over most of the Earth, and above 70 ppbv over some regions e.g. North America, western and central Eurasia, Brazil, East Asia and central and south-western Africa (Sitch et al 2007). O_3 is considered the most important pollutant in rural areas (Ashmore 2005). It not only affects human health (Iriti & Faoro 2008), but also affects materials and vegetation (Ashmore 2005). Evidence of its adverse effects on ecosystems is accumulating (for a review Fuhrer 2003; Ashmore 2005). Ren et al (2007), for instance, modelled data from four decades (1960s to 2000) in China and found that O_3 reduced soil and litter carbon storage and decreased the net primary productivity of terrestrial ecosystems. It is expected that in future elevated O_3 environments, many ecosystems will show reduced land-carbon storage accumulation, which will increase the accumulation of CO_2 in the atmosphere (Sitch et al 2007).

1.4.1 Formation, Abundance, and Trend of Ozone in the Troposphere

Tropospheric O_3 is formed from molecular oxygen through a series of photochemical reactions driven by ultraviolet radiation from the sun. It is formed by the photolysis of NO_2 (Fig. 2, reactions 1 and 2), which in turn, is formed by radicals (Fig. 2, reactions 3 and 4) resulting from the photooxidation of biogenic and anthropogenic VOCs (Atkinson & Arey 2003). Therefore, plant-emitted VOCs have an impact on the chemistry of the atmosphere.

$$NO_2 + h\nu \longrightarrow NO + O(^3P) \quad (1)$$

$$O(^3P) + O_2 + M \longrightarrow O_3 + M \quad (M = air) \quad (2)$$

$$RO_2\cdot + NO \longrightarrow RO\cdot + NO_2 \quad (3)$$

$$HO_2 + NO \longrightarrow OH + NO_2 \quad (4)$$

Figure 2. Chemical reactions leading to the formation of O_3 in the atmosphere according to Atkinson & Arey (2003)

O_3 shows daily, seasonal and spatial variation (Calogirou et al 1996). In general, higher O_3 concentrations are observed in spring or summer, depending on the latitude

and altitude (Vingarzan 2004). Currently, the abundance of this gas varies from 10 ppbv over tropical oceans to 100 ppbv in the upper troposphere. Highest concentrations can be found over polluted metropolitan areas. In northern latitudes the background O_3 can rise up to 80 ppbv (IPCC 2001).

Since the pre-industrial era, the abundance of this gas has increased 30% when globally averaged, and the prediction is that it will rise significantly throughout this century (IPCC 2001). In addition to the formation of O_3 from the reaction of biogenic and anthropogenic volatiles with natural NO_X, downward transport of stratospheric O_3 to the troposphere, contributes to an increase in background O_3 concentration (Vingarzan 2004). As well as metropolitan areas rural and remote areas are also endangered as a result of long-range transport of O_3 from polluted sites (IPCC 2001; Vingarzan 2004).

1.4.2 Effects of Ozone on Plants, Herbivores and Higher Trophic Levels

Both acute and chronic exposure to O_3 can cause damage to plants, although symptoms may differ. Acute exposure (120-500 ppbv) leads to the development of small necrotic lesions (Long & Naidu 2002). This symptom is the result of a genetically controlled programmed cell death which resembles the one occurring during incompatible plant-pathogen interactions (the hypersensitive response). In brief, once O_3 enters the mesophyll of leaves, there is an initial increase in ion fluxes (Jabs et al 1997) and accumulation of reactive oxygen species (ROS) e.g. superoxide anion or hydrogen peroxide in the apoplasm (Wohlgemuth et al 2002; Mahalingam & Fedoroff 2003). The O_3-induced ROS trigger signalling for the development and containment of the hypersensitive response, in which three hormones (salicylic acid, ethylene and jasmonic acid) are involved. Salicylic acid is produced early and has been found to be involved in the initiation of lesions or formation of the hypersensitive response (Rao & Davis 1999; Overmyer et al 2003). Within a few hours the biosynthesis of ethylene is also activated (Tuomainen et al 1997). This hormone seems to be involved in the propagation of the lesion (Overmyer et al 2000). Later, jasmonic acid is synthesised and accumulates at the borders of the lesions, correlating with the place where ethylene has previously accumulated (Tuominen et al 2004). Hence, it seems to be involved in the containment of lesion propagation by

reducing the magnitude of the oxidative burst and by interacting with the salicylic acid and ethylene pathways. In addition to programmed cell death, acute exposure of plants to O_3 can result in the development of systemic acquired resistance – SAR (Sharma et al 1996).

Chronic exposure of plants to O_3 (40-120 ppbv) can also result in small necrotic and chlorotic lesions. However, it is not uncommon for plants exposed to O_3 to show increased ROS scavenging systems, decreased photosynthesis capacity and accelerated senescence, without visible symptoms (Long & Naidu 2002). In addition, chronic exposure of plants to O_3 can also lead to changes in leaf chemistry. A recent meta-analysis by Valkama et al (2007) has assessed the results of 63 studies to understand the effects of O_3 on the leaf chemistry of forest species. The responses to O_3 exposure vary according to tree species, ontogenic stage, the type of O_3 exposure (e.g. indoor growth chambers, open top chambers, or in free air environment), and to a certain extent, to the duration of the O_3 exposure. With regard to primary metabolites, carbohydrates have been found to decrease in angiosperm species and saplings or to remain unaffected. The nutrient concentrations in some species e.g. *Betula pendula* Roth. and *Populus tremuloides* Michx. increase whereas in other birch species they decrease. However, a common response to O_3 seems to be an increase in carbon-based secondary metabolites, phenolics and terpenes.

The effects of O_3 on leaf chemistry have been less studied in herbaceous or non forest species. In soybean (*Glycine max* (L.)) leaves, reduced glucose and fructose contents have been observed at an O_3 concentration of 1,2 times ambient levels (Hamilton et al 2005), and in tobacco plants (*Nicotiana tabacum* L.) changes in the total amount of cembranoid diterpenes have been reported (Jackson et al 1999). In addition to changes in leaf chemistry, O_3 exposure induces the emission of VOCs, altering profiles (Wildt et al 2003; Loreto et al 2004; Vuorinen et al 2004) and might alter the properties of the plant surface (Müller & Riederer 2005).

From an agricultural point of view, O_3 can negatively affect crop yields due to its impact on photosynthesis. Decreased yields have been observed in wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.), and experimentally in tomato (*Lycopersicon*

esculentum L.), bean (*Phaseolus vulgaris* L.) and soybean (Ashmore 2005, for a review). In addition, the development of visible symptoms reduces the economic value of crops such as vegetables as their marketing depends on aesthetic appearance. The development of visible symptoms has been reported in bean, radish (*Raphanus sativus* L.) and turnip (*Brassica rapa* L.), and others in many parts of the world (Ashmore 2005). In Southern Europe, several crops have been found to be damaged by O_3 episodes, but watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) is especially affected by the development of visible symptoms (Pleijel 2000). Thus, O_3 can economically affect farmers in rural areas.

The changes in plants caused by O_3 can affect plant-insect interactions. Many studies have addressed the plant-mediated effects of O_3 on herbivore performance (Coleman & Jones 1988; Whittaker et al 1989; Bolsinger et al 1992; Holopainen et al 1997; Kopper & Lindroth 2003; Awmack et al 2004) and behaviour (Jones & Coleman 1988; Bolsinger et al 1992; Jackson et al 1999; Jøndrup et al 2002; Agrell et al 2005; Hamilton et al 2005). The direction and magnitude of the response varies between studies, which may be related to the insect guild (e.g. whether they feed on the foliage or on the phloem). The meta-analysis of Valkama et al (2007) found that the pupal mass of insect herbivores attacking forest species increases, and the development time is shortened under increased O_3 conditions. The relative growth rate of chewing insects is also increased. Such changes are dependent on the length of the O_3 exposure.

O_3 effects on herbivores can in turn affect the performance of higher trophic levels (Holton et al 2003) and their abundance in field conditions (Percy et al 2002). It can negatively affect the searching efficiency of parasitoids, as observed in *Asobara tabida* Nees (Hymenoptera:Braconidae) by Gate et al (1995). Although the causes are not known, the authors have proposed that the reduced searching efficiency can be due to: 1) direct effects on the functioning of olfactory receptors or the integration of receptor responses, 2) the reaction of O_3 with synomones, e.g phytogenic VOCs which attract natural enemies, as herbivore-induced volatiles comprise an array of O_3 reactive terpenes and GLVs, or 3) altered phytogenic VOC emissions. The latter is possible since O_3 is an abiotic elicitor of plant defences (Loreto et al 2004; Vuorinen et al 2004). In general, little has been done to assess the effects of O_3 on insect

chemical communication, in particular on tritrophic systems. There is some evidence that O_3 can degrade insect pheromones disrupting intra-specific communication (Arndt 1995). However, none of the studies have assessed the ecological consequences of the reaction of terpenes and GLVs with this pollutant.

1.5 Tritrophic Systems and Overview of the Experiments

1.5.1 Brassica oleracea – Plutella xylostella – Cotesia vestalis (=C. plutellae)

The indirect defence of *Brassica* species has been widely studied. Species of the genera *Cotesia* (Hymenoptera: Braconidae) have been shown to be attracted by cabbage (*Brassica oleraceae* ssp. *capitata* L.) (Vuorinen et al 2004a), oilseed rape (*Brassica napus* L.) (Potting et al 1999), and Brussel sprout (*Brassica oleraceae* L. ssp. *gemmifera* Zenker) (Scascighini et al 2005) when damaged by herbivores. The botanical family is characterised by the presence of a special group of secondary metabolites: the glucosinolates. Glucosinolates are degraded by myrosinase enzymes and some of the breakdown products are volatile. They are also induced after herbivore feeding (Agelopoulos & Keller 1994; Geervliet et al 1997). The diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), is an oligophagous species feeding on cabbage and relatives. From an agricultural point of view, it is an important widespread pest that causes significant yield losses around the world, and it has developed resistance to most of the pesticides used in the field (Sarfraz et al 2006 for a review). *Cotesia vestalis* (Haliday) (=*Cotesia plutellae* (Kurdjumov)) is a solitary endoparasitoid that oviposits in diamondback moth larvae. It is a specialist parasitoid, even though it has been reported to parasitise other herbivore species (Shiojiri et al 2000). Since *P. xylostella* feeds on a few plant species, and *C. vestalis* mainly parasitises *P. xylostella*, the tritrophic system shows a high degree of specialization.

1.5.2 Phaseolus lunatus – Tetranychus urticae – Phytoseiulus persimilis

In addition to the Class Insecta, the exploitation of herbivore-induced VOCs has also been reported for predatory mites (Dicke 1999). There is behavioural and chemical evidence that at least three species in the family Phytoseiidae use volatiles induced by

herbivory to locate their prey (Turlings and Wäckers 2004 for a review). The orientation of the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) to induced VOCs emitted by lima bean (*Phaseolus lunatus*) L. (Fabaceae) plants damaged by the two spotted mite (*Tetranychus urticae*) Koch (Acari: Tetranychidae) is well documented. This tritrophic system has been used as a model system to study indirect defence of plants since the early 1990s (Dicke et al 1990; Dicke & Dijkman 1992; Dicke et al 1993). The two spotted mite is a polyphagous pest. Its host range includes wild and cultivated plant species growing outdoors and in greenhouses, including perennial and annual plants in different plant families. Some of its main hosts include horticultural crops such as strawberry, eggplant and cucumber, deciduous trees such as apple, pear, and peach trees (Takafuji et al 2000), and ornamental plants such as *Gerbera* (Krips et al 2001) and ivy geranium (Opit et al 2004). The fact that the herbivorous mites feed on a wide range of plants makes this tritrophic system less specialised than the one described earlier (1.5.1). The herbivore-induced VOC blend varies among hosts (van den Boom et al 2004), and the predatory mite is thus challenged with learning variable VOC profiles to find its prey. *P. persimilis* is a specialist predator of spider mites from the genus *Tetranychus* (Krips et al 2001). It is a very efficient predator and is therefore an important component in integrated pest management programs for controlling spider mites on vegetables and ornamentals in greenhouses of Europe and North America (Weeden et al *online*).

1.5.3 The Experiments

This thesis consists of four experiments which are summarized in Table 1. In the first three experiments (Chapters 2-4), phytogenic VOCs were mixed with O_3-enriched air to assess the effects of different concentrations of this oxidant on constitutively-emitted and herbivore-induced VOC profiles. In the first experiment (Chapter 2), the formation of SOAs was determined in addition to the changes (relative proportions) on the VOC profile. The ozonolysis of phytogenic VOCs was conducted in a separate Teflon reaction chamber to avoid the effect of plant surfaces on SOAs formation (Fruekilde et al 1998; Müller & Riederer 2005) or the effect of O_3 on VOC emissions (Loreto et al 2004). O_3 levels were set at 100, 200 and 400 ppbv. Although these

concentrations are above realistic conditions, they have been used in several studies to assess the formation of SOAs (e.g. Hoffmann et al 1997). The main reason for using high O_3 concentrations is that a continuous flow reactor system was used, which has several advantages over classical chamber experiments, e.g., controlled mixing of O_3 and VOCs, controlled residence times for reactions, easy to vary concentrations, etc. However, the residence time (time for ozonolysis and particle formation and growth) is quite limited (ca. 17 min) compared to classical chamber studies (hours). Therefore, it was necessary that high O_3 concentrations were used in order to observe SOAs formation (formed particles have to grow to a particle size of 5 nm before they can be detected; the detection limit of the system was 5 nm).

In the following two experiments (Chapters 3 & 4), behavioural tests using the selected tritrophic systems were conducted in addition to VOC collection and analysis to evaluate the ecological consequences of VOC ozonolysis. In both of these experiments, the behavioural assays were conducted with a Y-tube olfactometer, which tests the olfactory orientation of insects in a binary choice system (Schoonhoven et al 2006). For both selected tritrophic systems, the preference of the natural enemies for either intact or herbivore-damaged plants was assessed at 0 (control), 60 and 120 ppbv O_3, which realistically represent O_3 episodes (Pleijel 2000) (Chapter 3). For the more specialised tritrophic system (1.5.1), the preference of the parasitoid for either herbivore-damaged plants at 0 ppbv or herbivore-damaged plants at 120 ppbv O_3 was also assessed (Chapter 4). Although comparing herbivore-induced VOCs in two different O_3 environments is an unrealistic situation, this approach allows a better understanding of the preferences of the parasitoid towards reactive terpenes and GLVs.

The VOC collection system and analytical methods for determining VOC emissions used for these experiments are described in detail in Chapter 2. For all the experiments, the quantities of VOCs are expressed in ng g^{-1} DW g^{-1} for emission, and in ng l^{-1} for the concentrations. The setup for the behavioural tests conducted under laboratory conditions is described in Chapter 3. The modification of this setup for comparing the preference of the parasitoid in two different O_3 environments is described in Chapter 4.

To support the laboratory observations, the behaviour of the parasitoid *C. vestalis* was assessed under ambient and double background O_3 in field conditions (Chapter 5). This experiment was conducted during two consecutive years (2005 and 2006) in the open-air O_3 fumigation system of the University of Kuopio, located in Ruohoniemi (62°13'N, 27°35'E, 80m.s.l.) (Wulff et al 1992; Karnosky et al 2007). This experiment coupled field observations of host searching by the parasitoids with quantification of their parasitism rate in two different O_3 environments.

1.6 Aims of the Study

This study was aimed at assessing how O_3 affects the relative proportions of compounds (in particular terpenes and GLVs) in VOC profiles from intact and herbivore-damaged plants by quantifying emitted compounds at different O_3 concentrations.

In addition, this study was aimed at assessing the ecological implications of oxidation of VOCs by assessing whether indirect defence of plants e.g. signalling between plants and higher trophic levels (predators and parasitoids) is disrupted under controlled conditions and field conditions.

The research questions to be answered by this study were:

1) Does the ozonolysis of phytogenic VOCs change the relative proportions of the VOC profile in the headspace of intact and herbivore-damaged plants?

2) Does O_3 degradation of common herbivore-induced VOCs (terpenes and GLVs) affect the orientation of natural enemies e.g. predators and parasitoids?

Table 1. Summary of the experiments and hypotheses tested in the original publications

Previous knowledge	Hypotheses tested	Methodology	Living material	Chapter
The lifetimes of terpenes and some oxygenated compounds are in the range of minutes to a few hours when reacting with O_3[a]. Gas/particle partitioning of products from the reaction O_3/terpenes [a,b].	O_3 leads to changes in the relative proportions of compounds in the VOC blend. O_3 quenching occurs beyond the boundary layer of the leaf. Plant species without terpene storage are potential sources of SOAs in the troposphere.	Exposure of phytogenic VOCs to 100, 200 and 400 ppbv O_3 pulses in a Teflon reaction chamber. Analysis of O_3 and VOCs (GC-MS) before and after the reaction chamber. Analysis of aerosol particle number size distribution (DMPS) at the end of the reaction chamber.	White cabbage plants (*B. oleracea* var. *capitata*) *P. xylostella* larvae	2
Terpenes and GLVs are major herbivore-induced compounds[c]. Natural enemies rely on herbivore-induced plant VOCs, including terpenes and GLVs[d,e,f].	O_3 affects the VOCs in the headspace of intact and herbivore damaged plants. Degradation of constitutive and herbivore-induced terpenes and GLVs by O_3 affects the orientation of natural enemies towards damaged plants.	Exposure of intact and herbivore-damaged plants to 0, 60 and 120 ppbv of O_3 during VOC collections and insect behavioural tests. Analysis of VOCs (GC-MS) in the headspace of intact and herbivore-damaged plants. Dual choice behavioural tests (Y-tube olfactometer).	White cabbage plants *P. xylostella* larvae *C. vestalis* females (parasitoid) Lima bean (*P. lunatus*) *T. urticae* *P. persimilis* (predator)	3

Previous knowledge	Hypotheses tested	Methodology	Living material	Chapter
Compounds other than terpenes emitted by *Brassica* spp. are attractive to natural enemies[g,h]. *P. xylostella* prefers herbivore-damaged plants over intact and mechanically-damaged plants[i]. Degradation of terpenes and GLVs by O_3 does not disrupt the orientation of *C. vestalis*[j].	Degradation of herbivore-induced terpenes and GLVs by O_3 does not decrease the preference of a foraging natural enemy for damaged plants.	Exposure of intact and herbivore-damaged plants to 0 and 120 ppbv of O_3 during VOC collections and insect behavioural tests. Analysis of VOCs (GC-MS) in the headspace of herbivore-damaged plants. Dual choice behavioural tests (Y-tube olfactometer).	Cabbage plants *P. xylostella* larvae *C. vestalis* females	4
In field O_3-enrichment experiments herbivore densities increased[k] . In field O_3-enrichment experiments parasitoid densities decreased[k].	In field conditions, elevated O_3 affects the searching behaviour of natural enemies, which results in reduced parasitism rates.	O_3 exposure of herbivore infested plants in open-air O_3 fumigation system targeted to double ambient O_3. Field observation of the number of wasps in the headspace of infested plants.	Cabbage plants *P. xylostella* larvae *C. vestalis* females	5

[a] Atkinson & Arey (2003), [b] Moukhtar et al (2005), [c] Paré & Tumlinson, 1999, [d] Dicke (1999), [e] Dicke et al (1990), [f] Reddy et al (2002) , [g] Agelopoulos & Keller (1994), [h] Mattiacci et al (1994), [i] Shiojiri et al (2006), [j] Pinto et al (2007), [k] Percy et al (2002)

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CHAPTER 2

The effects of increasing atmospheric ozone on biogenic monoterpene profiles and the formation of secondary organic aerosols

Pinto DM, Tiiva P, Miettinen P, Joutsensaari J, Kokkola H, Nerg A-M, Laaksonen A, Holopainen JK. 2007

Atmospheric Environment 41: 4877-4887

CHAPTER 3

Ozone degrades common herbivore-induced plant volatiles: does this affect herbivore prey location by predators and parasitoids?

Pinto DM, Blande JD, Nykänen R, Dong, WX, Nerg A-M, Holopainen JK (2007)

Journal of Chemical Ecology 33: 683-684

CHAPTER 4

The role of ozone reactive compounds, terpenes and green leaf volatiles (GLVs), in the orientation of *Cotesia plutellae*

Pinto DM, Nerg A-M, Holopainen JK (2007)

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CHAPTER 5

Host location behavior of *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) in ambient and moderately elevated ozone in field conditions

Pinto DM, Himanen S, Nissinen A, Nerg A-M, Holopainen JK. 2008

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CHAPTER 6

General Discussion

CHAPTER 6: General Discussion

6.1 Ozonolysis of terpenes and GLVs

Irrespective of the set-up used, O_3 concentration, herbivore treatment (intact or damaged plants) or plant species, all the terpenes except 1,8-cineole (only emitted by cabbage plants), and all the GLVs, were partially or totally decomposed by O_3 (Chapters 2 to 4). This is the result of variation in the number of C-C double bonds among the compounds (Calogirou et al 1996). This significantly affected the relative proportions of compounds in the VOC blend as shown in Chapter 2. In this experiment, there was degradation of all other monoterpenes at the highest O_3 treatment (400 ppbv), resulting in a profile dominated by 1,8-cineole. It is not surprising that 1,8-cineole was not degraded by O_3 as it is a saturated aliphatic monoterpene. Calogirou et al (1996) found that this compound remained unaffected during sampling on Tenax TA, which was also used as trap adsorbent during all the VOC collections in this study. The lifetime of this compound has been estimated to be over 110 days when reacting with O_3 in the atmosphere (Atkinson & Arey 2003). Interestingly, in the experiments described in Chapters 3 & 4, the concentrations of 1,8-cineole increased significantly at 60 and 120 ppbv, compared to those in filtered air, which might be the result of differences in the set-ups or in the VOC collection time, since they differed to those in Chapter 2.

Not all compounds were significantly degraded, and some compounds varied in the extent that they were detected by GC-MS even when measured at the same O_3 treatment and using the same set-up (Chapter 3 & 4). The ozonolysis of terpenes is influenced by many factors. Pommer (2003) found that the reactions of α-pinene and Δ-3-carene with O_3 in a dark reaction chamber are influenced by O_3 level, relative humidity and reaction time, among other factors. It can also be influenced by the interaction between O_3 and relative humidity (Fick et al 2002). In addition, the oxidation of terpenes by O_3 is a source of OH· (Pfeiffer et al 1998; Aschmann et al 2002), which are also able to react with terpenes (Atkinson & Arey 2003). The production of OH· is dependent on the terpene concentration, and an increasing function of the O_3 concentration for mixing ratios up to 200 ppbv (Pfeiffer et al 1998). The factors mentioned above might also have influenced the degradation of GLVs.

For example, the lifetime for (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate has been estimated to be 6,2 and 7,3 hours when reacting with O_3, but it is only 1,3 and 1,8 hours when reacting with OH· (Atkinson & Arey 2003).

The variation in reactions by a single compound are likely to be explained by the fact that biogenic VOC emissions were used and therefore, the ozonolysis of terpenes took place in complex mixtures at different emission rates. Moreover, there might have been variation in the relative humidity among experiments. It is possible that one of the main factors affecting the reactions in the experiments described in Chapters 3 & 4 was the duration of the reaction (Pommer 2003; Pommer et al 2004). In both experiments, the same setup was used but in Chapter 3, plants were exposed to 60 ppbv before increasing the O_3 concentration to 120 ppbv, whereas in Chapter 4, O_3 was increased straight from 0 to 120 ppbv.

Herbivore feeding also induces the phytogenic emission of compounds other than terpenes and LOX products. The family Brassicaceae, to which cabbage plants belong, is characterised by the production of glucosinolates, which are a sulfur-based group of secondary metabolites. Glucosinolates are hydrolyzed by thioglucosidase (myrosinase) enzymes. Such hydrolysis is facilitated by rupturing of tissue (for example due to herbivore feeding) and results in the production of organic isothiocyanates, thiocyanates and nitriles, many of which are relatively small volatile molecules (Tsao et al 2002; Yan & Chen 2007). Hence, *P. xylostella* feeding also induces the emission of nitriles, isothiocyanates and sulfides from cabbage plants (Agelopoulos & Keller 1994; Mattiacci et al 1994; Geervliet et al 1997). Since the glucosinolate breakdown products are a distinctive group of volatiles emitted by plant species of the order Brassicales, the emissions and concentrations of these compounds were quantified, in addition to terpenes and GLVs. Allyl nitrile and methyl thiocyanate, were emitted by both intact and damaged cabbage plants, while benzyl cyanide (BC) was the only compound induced by *P. xylostella*. None of these compounds were significantly degraded by O_3 (Chapter 3 & 4). Although to our knowledge their lifetimes in O_3 and/or OH· polluted environments have not been quantified, these results suggest that they are stable compounds in the atmosphere.

Mite feeding on Lima bean also elicits the emission of phytogenic compounds other than terpenes and GLVs, such as 2-butanone (ketone) and methyl salicylate (MeSA) (ester) (De Boer et al 2004). These two compounds were also not degraded by O_3. The lifetime of MeSA in the O_3 polluted atmosphere has been estimated at approximately 4 years (Canosa-Mas et al 2002). Interestingly, the emissions of MeSA were significantly increased by higher O_3 concentrations (Chapter 3). It is possible that a synergistic effect of O_3 and mite feeding occurred.

6.2 Formation of SOAs by cabbage plants

Few studies have measured the particle formation directly from biogenic emissions (e.g. Joutsensaari et al 2005; van Reken et al 2006). The potential of cabbage plants for forming SOAs was investigated in Chapter 2. The ozonolysis of monoterpenes emitted by cabbage plants resulted in the formation of SOAs, suggesting that species that do not store monoterpenes can be a source of particles in the atmosphere. However, at the lowest O_3 concentration (100 ppbv) few particles were observed. Sesquiterpenes have been found to have the potential to produce significantly more particles than monoterpenes (Bonn & Moortgat 2003). Van Reken et al (2006) found that exposure of two forest species to 50 ppbv O_3 resulted in particle formation, and the oxidation of *Pinus taeda* L. resulted in more particles, which might owe to the presence of sesquiterpenes in the VOC blend. Joutsensaari et al (2005) found that the ozonolysis of MeJA-induced VOCs from cabbage plants can form nanoparticles in growth chambers. In their experiment, the most abundantly emitted compounds were limonene, sabinene, (*E*,*E*)-α-farnesene, and DMNT. In the experiment of Chapter 2, sesquiterpenes and homoterpenes were not detected, although (*E*,*E*)-α-farnesene and DMNT are commonly induced by herbivory (Vuorinen et al 2004a). It is possible that the use of a Teflon filter before the reaction chamber to remove any stomatal wax particles, removed these less volatile compounds. Therefore, few particles were formed.

6.3 Behavioural responses of natural enemies

Inducible terpenes can dominate the herbivore-induced VOC blend from many plant species (Turlings & Ton 2006). They have been acknowledged as important olfactory cues for carnivorous arthropods (Dicke et al 1990). It has been shown that genetically engineered terpene over-expressing mutants attract carnivorous arthropods (Kappers et al 2005; Schnee et al 2006). GLVs can also dominate the herbivore-induced VOC blend although they are also emitted in response to other stresses such as wounding. They have also been found to evoke electrophysiological and behavioural responses in natural enemies (Reddy et al 2002; Smid et al 2002). In this study, the results of experiments conducted in laboratory conditions show that the parasitoid *Cotesia vestalis* and the predatory mite *Phytoseiulus persimilis* prefer the odour of plants damaged by their respective host and prey even after partially degrading these reactive compounds at 60 and 120 ppbv O_3 (Chapter 3). However, *C. vestalis* prefers the odour of damaged plants in filtered air over damaged plants in elevated O_3 (120 ppbv) (Chapter 4). In field conditions, the host finding behaviour and the parasitism rate of *C. vestalis* was not affected by elevated O_3 (Chapter 5).

The results of the experiments in Chapters 3 & 5 suggest that O_3 episodes, and future O_3-enriched environments will not disrupt the orientation of natural enemies. In cabbage plants, we found that glucosinolate breakdown products remained relatively unaffected by O_3. These compounds do not only act in induced direct defence of plants by repelling generalist herbivores (Renwick 2002), but are also important kairomones for specialist herbivores (Mewis et al 2002; Barker et al 2006), since volatile breakdown products can be emitted in low amounts under normal conditions (Tollsten & Bergström 1988 cited by Mewis et al 2002; Schoonhoven et al 2006). Natural enemies need to rely on odours that are most associated with their host or prey (Turlings & Wäckers 2004). Hence, it is likely that the specialist parasitoid *C. vestalis* has learned to exploit these *Brassica*-specific volatile compounds, as suggested by Agelopoulos & Keller (1994). For instance, the parasitoid *Diaeretiella rapae* (McIntosh) (Hymenoptera: Braconidae), specialised on the crucifer specialising aphid *Lipaphis erysimi* (Kaltenbach) orientates toward 3-butenyl isothiocyanate (Blande et al 2007), and *Pieris rapae*-induced nitriles might also serve as attractants for *Cotesia*

rubecula (Marshall) (van Poecke et al 2001). Indeed, there is some evidence that *C. vestalis* can also exploit glucosinolate breakdown products present in volatiles from the frass of host larvae (Reddy et al 2002).

In two experiments (Chapters 3 & 4), benzyl cyanide (BC) was induced by *P. xylostella* feeding. Other glucosinolate breakdown products were also detected (allyl nitrile and methyl thiocyanate), but in contrast to BC, traces of these two compounds were also found in the headspace of intact plants. It is likely that BC offered reliable information to *C. vestalis* after the increase in O_3 levels. In Brussels sprouts, Scascighini et al (2005) found that *P. brassicae* also induces the early emission of BC, and the preference of *C. glomerata* (L.) to damaged plants increases steadily during the first 3 hours after damage onset.

The results in Chapter 3 with the tritrophic system *Brassica oleraceae-Plutella xylostella-Cotesia vestalis* support the views of Mattiacci et al (1994) and Scascighini et al (2005) who suggested that terpenes may not contribute significantly to the blend of VOCs emitted by herbivore-damaged cabbage plants. Their relative proportion in the induced VOC blend is small, as other compounds are greatly increased. However, the results in Chapter 4 show that the partial ozonolysis of reactive terpenes and GLVs make the herbivore-induced VOC blend less attractive to the wasp. Hence, changes in the ratios (Bruce et al 2005; Schoonhoven et al 2006) decrease the preference of *C. vestalis* towards damaged plants. It is possible that the orientation of wasps was based on quantitative differences in compounds between the two odour sources. Shiojiri et al (2001) suggested that differences in the emission of the herbivore-induced homoterpene DMNT from *P. xylostella*-damaged plants can offer *C. vestalis* enough information to discriminate between plants infested by its host. Therefore, higher concentrations of this inducible homoterpene at 0 ppbv might have increased the preference of the wasps. In addition, Ibrahim et al (2005) found that *C. vestalis* orientates towards the constitutively-emitted terpene limonene. This compound was the only constitutively-emitted terpene that increased significantly after herbivore feeding (Chapter 4).

It is not known to what extent GLVs play a role in the orientation of wasps. Individual GLVs are attractive to *C. vestalis* (Reddy et al 2002), and they are significantly

increased during the first 5 hours of feeding by *P. brassicae* on Brussels sprout (Scascighini et al 2005), which seems to correlate with the orientation of *C. glomerata* (however the authors found that BC and a cineole are also increased within few hours of feeding onset). Generalist parasitoids such as *C. glomerata* that can parasitise crucifer-feeding herbivores, can orientate toward volatile products of the LOX pathway (Shiojiri et al 2006). However, more specialised parasitoids such as *C. vestalis* seem to rely on compounds specifically induced by herbivore feeding, in addition to GLVs (Potting et al 1999; Shiojiri et al 2006). In herbivorous insects, synergism of GLVs and terpenes with other compounds and pheromones (Schoonhoven et al 2006 and references therein; Tasin et al 2007) as well as redundancy (Tasin et al 2007) have been observed. It is possible that these phenomena also occur in insects from higher trophic levels. These results also showed that it is unlikely that the wasps relied on oxidation products resulting from the reactions between terpenes or GLVs with O_3 in Chapter 3.

In contrast to *C. vestalis*, *Phytoseiulus persimilis* is challenged to find its prey on a wide variety of plant species in nature. Its prey, *Tetranychus urticae*, is a generalist herbivore that feeds on a wide range of plants. Therefore, it is likely that this predatory mite relies more on common emitted herbivore-induced VOCs such as terpenoids. Indeed, there are many studies that have shown or suggest that inducible terpenoids elicit a behavioural response in *P. persimilis* (for example Dicke et al 1990; Krips et al 2001; Vuorinen et al 2004b; Kappers et al 2005). However, *T. urticae* also induced the emission of non terpenoid compounds such as MeSA and 2-butanone, which were not degraded in elevated O_3 levels (Chapter 3). The role of these compounds in the orientation of *P. persimilis* has been studied by De Boer et al (2004). Individually, they seem to be highly attractive to predatory mites reared on lima bean plants. Hence, it is likely that the relatively high concentrations of these two compounds at elevated O_3 levels might have been enough to allow the orientation of the predatory mites. At least to MeSA, the predatory mites respond in a dose-dependent way (De Boer & Dicke 2005). The high concentrations might have been influenced by the elevated number of mites per leaf used for infestation. However, higher infestations have been used by other authors (Arimura et al 2001).

6.4 VOCs in present and future ozone environments and tritrophic interactions

Future O_3-enriched conditions will alter phytogenic VOC profiles, but the emission of less reactive compounds that also act as semiochemicals such as BC, MeSA and 2-butanone, may allow orientation of predators towards their prey or host habitat. There is some evidence that the searching efficiency (host attacked per unit of search time) can be negatively affected by elevated O_3 which leads to reduced parasitism rates (Gate et al 1995). In the field experiment, no differences in the parasitism rate of *C. vestalis* were found between O_3 treatments, but the searching efficiency was not investigated (Chapter 5). It is more likely that a reduced searching efficiency is the result of O_3-mediated changes on the surfaces of leaves or substrate (Müller & Riederer 2005) rather than changes in VOC profiles. However, the ozonolysis of terpenes and GLVs cannot be discarded as a possible cause. For flying herbivorous insects, VOCs are mainly used during the early steps of the host-plant selection process, but olfactory cues in addition to contact cues might be exploited after contact with the plant (Schoonhoven et al 2006). For predatory mites, VOCs are used to locate spider-mite patches in a habitat and colonies within a patch (Dicke et al 1998). In contrast with O_3 episodes, in which elevated O_3 concentrations are reported over a few hours or days, the gradual increase of O_3 in the troposphere will offer natural enemies the possibility to adapt and to learn new odours and exploit different VOCs (or new ratios among compounds). Wasps and predatory mites are able to learn to associate a specific volatile with the presence of a host (Meiners et al 2003; De Boer & Dicke 2005 for a review). Moreover, the fact that other types of cues can also be exploited (for example visual cues) (Schoonhoven et al 2006) will increase their possibility of success at finding a host or prey.

Under current climate conditions, O_3 and other oxidants (e.g. $OH\cdot$ and $NO_3\cdot$) are present in the troposphere. Many factors such as plant physiological state, wind speed and direction and temperature affect the distribution and concentration of VOC blends from plants (Schoonhoven et al 2006). Atmospheric oxidants that react with plant-emitted VOCs cannot be excluded. The composition of the odour plume that natural enemies utilise in nature might vary with distance from the plant due to differences in the reactivity of VOCs. It is likely that high concentrations of terpenes, for example,

are present in the boundary layer of the leaf (Holopainen 2004), and less reactive compounds can be sensed by insects from a distance. It is possible that environmentally stable compounds allow the orientation of natural enemies over long ranges, while terpenes and GLVs might add information of host presence over short ranges (Chapters 3 & 4).

6.5 Methodological considerations and limitations

Standard methods have been used for VOC collection and behavioural tests (Vuorinen et al 2004a,b; Ibrahim et al 2005). It is worth noting that the analytical equipment used for VOC analyses is not as sensitive as the chemoreceptors of arthropods themselves (Pickett et al 1998), and therefore the interpretation of the results is based on those compounds detected in the analyses. For the behavioural tests, in contrast to many studies, in which roots have been pruned (e.g.Vuorinen et al 2004a) or leaves detached (e.g.van den Boom et al 2004) we used potted plants as odour sources. This avoided changes in the VOC profile as a result of mechanical damage (Vuorinen et al 2005).

Since VOCs were collected in O_3-enriched air, O_3 scrubbers made from potassium iodide (KI) were used for all collections to prevent reaction with the adsorbent. KI-based O_3 scrubbers have been shown to be efficient at scavenging O_3 (Helmig & Greenberg 1995 and references therein). They were tested before each experiment and shown to remove 100% of O_3. The recovery of terpenes when working with KI-based scrubbers has been estimated to be higher than 95% (Fick et al 2001). It is possible that the O_3 scrubber in the sampling lines might have slightly altered the results. However, the fact that they were used in all collections would have reduced this effect.

When O_3 was delivered to two odour sources (Chapter 3), there were differences in the O_3 concentration between the two odour sources. In general, lower O_3 concentrations, indicating higher O_3 consumption, were observed in the vessels containing herbivore-damaged plants, which may correspond with higher VOC emissions by the injured plants and therefore result in higher O_3 quenching

(Holopainen 2004). However, the O_3 scavenging might also have been influenced by differences in humidity and plant leaf area due to dry deposition (Kerstiens & Lendzian 1989; Kurpius & Goldstein 2003; Holopainen 2004). The target concentrations were adjusted relative to the vessel containing intact plants, as they were expected to be physiologically less variable among individuals (Chapter 3). It would have been interesting to develop a system, in which reactions took place in a separate chamber without contact with plants.

Predatory mites are able to orientate towards herbivore-damaged lima beans even when emissions of MeSA, a product of the shikimic acid pathway, are not detectable (Vuorinen et al 2004b). This suggests that this compound is not the only one that drives the orientation of *P. persimilis* towards its prey. Since there is evidence that this predatory mite uses terpenoids as olfactory cues, it would be interesting to assess how O_3 affects the orientation of *P. persimilis* towards an herbivore-damaged plant other than Lima bean, or towards JA-induced plants. The tritrophic system was chosen for this study because it has previously served as a model system (De Boer & Dicke 2005 and references therein); however, not all plant species infested by *T. urticae* emit MeSA (e.g. eggplant) (van den Boom et al 2004). JA-induced VOC blends also (e.g. gerbera) lack this compound, and are able to attract the predatory mite (Gols et al 1999). Different levels of infestation may also affect the results.

Similarly, it would be interesting to test other tritrophic systems. For example, *Spodoptera littoralis*-damaged maize plants emit many induced terpenoids and GLVs. Indole is also induced, but shikimic acid derivatives seem not to play a role as synomones for *Cotesia marginiventris* (Cresson) and *Microplitis rufiventris* (Kok.) (D'Alessandro et al 2006). This suggests that terpenoids and GLVs, which are reactive compounds, might be the most important synomones for the wasps.

Due to their molecular composition, insect pheromones can also be prone to degradation by O_3 (Arndt, 1995). It would be interesting to test insect pheromones. For example, the alarm pheromone of many aphid species consists of the sesquiterpene (*E*)-β-farnesene. The degradation of this compound could affect intra- and interspecific interactions (Beale et al 2006).

The results of the field experiment (Chapter 5) support the laboratory observations for the tritrophic system *B. oleracea-P. xylostella-C. vestalis* (Chapter 3). However, it is worth pointing out that the O_3 concentration was targeted at double the background O_3 concentration, but with the exception of a few hours per day during the whole experiment, the O_3 concentrations were relatively low. In addition, this experiment included visual information that may have helped the wasps locate damaged plants. Most of the previous studies addressing the effects of O_3 on plant-insect interactions in the field have been conducted with perennial species, in which an accumulative effect of O_3 occurs. The accumulated dose over a threshold of 40 ppbv (AOT40) is the sum of the differences between the hourly mean O_3 concentration (in ppbv) and 40 ppbv for each hour when the concentration exceeds 40 ppbv, accumulated during daylight hours (Fuhrer et al 1997). Percy et al (2002) found that the abundance of natural enemies is negatively affected by elevated O_3 on tree species. It is probable that in that experiment other plant-mediated factors have been involved.

6.6 Conclusions

The answer to the addressed questions:

1) Does the ozonolysis of phytogenic VOCs change the relative proportions of the VOC profile in the headspace of intact and herbivore-damaged plants? YES. O_3 quenching and ozonolysis of VOCs is not limited to the boundary layer of the leaf. This leads to altered ratios among individual compounds in the VOC profiles emitted by intact and herbivore-damaged plants.

2) Does O_3 degradation of common herbivore-induced VOCs (terpenes and GLVs) affect the orientation of natural enemies e.g. predators and parasitoids? Tritrophic signalling is not affected by the ozonolysis of VOCs. The herbivore-induced VOC blend includes O_3-stable compounds that natural enemies can use. However, the degradation of reactive VOCs decreases the preference of *C. vestalis*.

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